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CHOLESTEROL DEPRESSANT

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Title of the Invention: Cholesterol depressant

Abstract: A cholesterol depressant comprising a water-insoluble fraction obtained by removing a water-soluble fraction from an enzymatically decomposed protein which is soluble in a SDS (sodium dodecyl sulfate) solution, and which shows a molecular weight of 1,000 to 150,000 after being dissolved in the SDS solution.

Details of the Invention

Technical field: The invention is about the cholesterol depressant.

Technical background: As the diet is very effective in the prevention and improvement of cholesterol level, the risk factor in arteriosclerosis there have been many studies made on how to control serum cholesterol level by dietary proteins and it was learned that compared to animal proteins like casein, plant proteins like soy proteins are much more effective in the lowering of serum cholesterol level. Also, it is known that of the enzymatic hydrolysates of proteins peptides of certain specific molecular weights can lower cholesterol level (Japanese Patent Publication Sho 60-11425). However, there has been no reports available on the cholesterol depressant from the hydrophobic polymers of the enzymatic hydrolysates of proteins.

Details

In the study of the effects of dietary proteins on the serum cholesterol level for the prevention and improvement of cholesterol reduction in serum by diet the inventors learned that compared to the animal proteins like casein the plant proteins like soy proteins can lower serum cholesterol better, and that peptides of certain molecular weights are more

effective (Japanese Patent Publication Sho 60-11425). The inventors therefore carried out studies on the effects of these peptides on the chlolesterol metabolism and the mechanism (s) of lowering cholesterol level and were convinced that of the enzymatic hydrolysates of these some fraction(s) should have the cholesterol-lowering effects and searched for such fractions.

The inventors worked toward this line of thought and while studying the cholesterol lowering effects of the various fractions of the enzymatic hydrolysates found that the precipitate fraction, resulting from the enzymatic hydrolysis of the proteins (insoluble in water but soluble in sodium dodecyl sulfate solution) had prominent cholesterol lowering effect and thus came up with the invention. The invention is therefore about the water-insoluble fraction separated from the water-soluble fraction of the protein hydrolysates. The fraction is soluble in SDS (sodium dodecyl sulfate) solution and after dissolution in SDS solution the material(s) has the molecular weight of 1,000-150,000 and has the cholesterol-lowering effect.

The insoluble fraction which constitutes the cholesterol-lowering substance claimed in the invention is water-insoluble. It is usually insoluble in water at the pH range of 2-8.

The water-insoluble fraction which comprises the cholesterol-lowering substance of this invention is soluble in surfactant solutions like SDS. In other words, this water-insoluble fraction is a hydrophobic material with hydrophobic structure(s). Therefore, the cholesterol-lowering substance of this invention contains the water-insoluble fraction (hydrophobic polymers) consisting of hydrophobic part of the protein hydrolysate which is obtained after removal of the hydrophilic fraction(s) of the enzymatic hydrolysates of the proteins.

The water-insoluble fraction consistuting the cholesterol-lowering substance(s) will have molecular weights ranging from about 1,000-150,000 when they are solubilized in 1% SDS solution (pH 6.9). Thus, the enzymatic hydrolysates of molecular weight of about 1,000-150,000 (preferably 2,000-50,000) constitute the insoluble fraction of the enzymatic hydrolysates as such or by polymerization and such a water-insoluble fraction has excellent cholesterol-lowering effect. Also, the cholesterol-lowering substance of this invention is (are) hydrophobic polymer(s) with excellent hydrophobic structure(s) with hydrophobic amino acid (Try, Phe, Val, Ile, Leu and Trp) composition over 30 weight % (preferably over 35 weight %). For example, compared with casein the soy proteins can lower cholesterol better but the water-insoluble fraction obtained by the enzymatic hydrolysis of soy proteins should have the molecular weight of about 1,000-150,000 (preferably 2,000-50,000)

when solubilized in 1% SDS solution (pH 6.9). Its high performance liquid chromatography (HPLC) pattern shows, for example, main peaks at about 8,600, 7,400 and 2,500, and/or about 86,000, 25,000, 7,600 and 2,900 (thus in the range of 2,000-about 100,000). Also, the proportion of hydrophobic amino acids (Tyr, Phe, Val, Ile, Leu and Trp) should be over 30 weight % (preferably over 35 weight %).

As described above, the cholesterol-lowering substance of this invention consists of the water-insoluble fraction of the protein hydrolysates which show cholesterol-lowering effects. It can be served in the forms of liquid (suspension) and dry powder. It can be made into tablets with an excipient. If necessary, it can contain other protein(s), carbohydrate(s), fiber(s), vitamin(s), minerals and other excipient(s). It can also be used as the ingredient in the diet. As it comes from natural proteins and there is no side effects from excess intake it can be used according to the serum cholesterol level.

Next, preparation of the cholesterol-lowering substance of this invention will be explained. It can be prepared by separating the water-insoluble fraction from the enzymatic hydrolysates of the protein which is carried out in aqueous system.

Proteins used should have lots of hydrophobic regions. The hydrophobic regions of the protein is hydrolyzed by enzyme(s) in aqueous system and the insoluble fraction obtained after the water-soluble fraction(s), which has low molecular weight peptides, is removed. Proteins with lots of hydrophobic regions can be an animal protein like serum proteins, egg white, poultry, fish, shell-fish or lactoalbumin, etc. or a plant protein like soy proteins, peanut proteins, oil seed proteins, gluten or glutenin or microbial proteins but according to the invention a plant protein like soy protein is richer in water-insoluble fraction(s) than the animal protein like casein for cholesterol-lowering effect.

Enzymatic hydrolysis can be done in any way so long as it results in hydrophobic polymers. Animal proteases like pepsin, trypsin and pancreatin, plant proteases like papain, ficin and promelein, etc. fungal proteases from Aspergillus, bacterial proteases from Bacillus and other microbial proteases can be used in the hydrolysis. The protease can be either acid, neutral or alkaline protease. It can be either endoprotease or exoprotease but combination of endo- and exoproteases is preferred becasue it can give the insoluble fraction with less bitter taste.

Conditions for enzymatic hydrolysis can be any (temperature, pH, E/S ratio, time and concentration, etc.) so long as more insoluble fraction(s) can be obtained. For example, the substrate concentration depends on the protein and enzyme but is better to be higher than

its optimum concentration for hydrolysis which may skew the optimum temperature and pH for hydrolysis. For example, in the case of soy protein 3-20% is better.

Next, the protein is hydrolyzed by enzyme in an aqueous solution and the resultant water-insoluble fraction can be separated. Usually the water-insoluble fraction which precipitates in neutal (aq.) solution is collected. Depending on the conditions for the treatment of the enzyme hydrolysates the insoluble fraction(s) resulting at pH 2-8 is separated.

Fractionation can be done by known means for the separation of insolubles such as centrifugation and filtration. Filtration can be done, for example, with a filter press or precise filtration.

In addition, the insoluble fraction may be contaminated by some soluble fraction(s) in operations but the insoluble fraction claimed in this invention can include some soluble fraction(s) which may contaminate the insoluble fraction in the fractionation. If necessary, the contaminants can be removed by repeated washing in water for purer insoluble fraction.

The insoluble fraction resulting from the fractionation is enzyme-inactivated and if necessary is neutralized and used as the cholersterol-lowering substance, but usually it is dried (by lyophilization, atomization, etc.) to powder and used as such.

The cholesterol-lowering substance of the invention contains the said insoluble fraction(s) as the effective component for cholesterol-lowering.

As described above, by this invention it becomes possible to prepare the cholesterol-lowering substance which can reduce serum cholesterol level. Examples will be given below to further explain the invention.

Example 1:

One hundred weight parts (to be called parts thereafter) (Fuji Pro NBW-R, Fuji Seiyu Kabushiki Kaisha) was dissolved in water to make a 10% solution (pH 7). One part each of protin FN (Yamato Kasei Kabushiki Kaisha, an *Aspergillus oryzae* protease) and protin AC (Yamato Kasei Kabushiki Kaisha, *Bacillus subtilis* protease) were added and enzyme hydrolysis was carried out at 50 °C for five hours. The hydrolysate solution was centrifuged (5,000 rpm x 20 min.) and the precipitate was collected. About two volumes of water was added to the precipitate and the mixture was washed by stirring, again centrifuged and the re-

sultant precipitate was heated at 80 °C for 30 minutes to inactivated the enzymes. After lyophilization 30 parts of cholesterol-lowering substance was obtained.

Next, the starting material, separated soy protein (SPI), the cholesterol-lowering substance (HMF) and purified HMF prepared by washing with 10 volumes of water five time were analyzed for their amino acid compositions by HCl hydrolysis and with an amino acid analyzer. Results are shown in Table 1.

Table 1 (Unit in weight %)

Amino acid		SPI	HMF	Purified HMF
Thr		3.8	4.0	4.2
Tyr	*	3.0	4.5	3.5
Phe	*	5.3	8.9	7.0
Cys		1.3	1.9	1.4
Met		1.4	1.4	1.5
Val	*	4.8	8.3	7.0
Ile	*	4.9	6.5	7.1
Leu	*	8.2	10.5	11.5
Lys		6.4	4.7	4.7
Trp	*	1.4	2.8	2.0
His		2.8	2.4	2.3
Asp		11.9	9.8	9.9
Ser		5.1	4.9	5.0
Glu		20.8	12.4	11.1
Pro		5.7	4.4	4.3
Gly		4.2	4.4	4.8
Ala		4.2	5.2	5.8
Arg		7.5	6.1	7.0

From these data it was learned that while the hydrophobic amino acid composition (*) of SPI was 27.6 weight% in HMF it was 37.5 weight % and 38.1 weight % in the purified HMF, showing that the proportion of these hydrophobic amino acids increased in HMF and the purified HMF.

Next, a high cholesterol diet containing HMF (Table 2) and a high cholesterol diet containing SPI were administered for 24 days to the five weeks old Sprague-Dawley male rats (body weight, 105-108 gm, 8-9 rats per group). The animals were then sacrified and blood collected and body dissected and their serum liver cholesterol was determined. Results are shown in Table 3.

From these results it was clear that HMF exhibited very prominent serum cholesterol lowering effect.

Experiment 1:

Table 2. High cholesterol diet

Protein	20 gm
Fat	10 gm
Water-soluble vitamin mixture	1 gm
Salt mixture	4 gm
Chlorinated choline	0.15 gm
Cellulose powder	2 gm
Vitamin A	400 ug
Vitamin D	5 ug
Tocopherol	10 mg
Cholesterol	0.5 gm
Sucrose	Trace
	Sum= 100 gm

Table 3

Group	Cholesterol level Serum (mg/dl)	Liver (mg/gm)
SPI	340±22	69.5±2.7
HMF	99.4±6.6 *1	7.70±0.97 *2

*1: Significant difference vs. SPI. p small-than 0.01
*2: Significant difference vs. SPI. p small-than 0.05.

The cholesterol-lowering substance (HMF) prepared as in Example 1 was dissolved in 1% SDS (sodium dodecy sulfate) and its molecular weight distribution pattern was studied by HPLC (TSK gel 3000SW; column, 7.5 mm x 50 cm). As markers bovine serum albumen (BSA, MW 67,000), soy trypsin inhibitor (MW 20,000), insulin B chain (MW 3500) and pentaglutamic acid (MW 664) were used. For elution SDS-containing 0.025 M phosphate buffer (ph 6.9) was used. Fractionation was done at the flowrate of 0.8 ml/min. Detection was done at UV 280 nm. Absorbancy at 280 nm was determined. Fig. 1 shows the HPLC chromatographic pattern. This shows that HMF had major peaks at MW 85,000, 7,400 and 2,500 including some peaks of MW 120. The MW range was about 1,000-150,000.

Experiment 2:

Purified HMF was run through HPLC as in Experiment 1. Results are shown in Fig. 2. It showed major peaks at MW 85,000, 7,400 and 2,500 with a small peak at MW 120. The MW range was about 1,000-150,000.

Example 2:

As in Example 1 a high cholesterol diet, shown in Table 4, was given to Sprague-Dawley

male rats (six per group) for 14 days with the protein serving as 20% of the nitrogen source, and as in Example 1 their serum cholesterol level was determined by the enzymatic method. Results are shown in Table 5.

Table 4

No.	Protein composition (wt. ratio)
1	HMF : Casein = 100: 0
2	HMF : Casin = 75: 25
3	HMF : Casein = 50: 50
4	HMF : Casein = 20: 80
5	HMF : Casein = 10: 90
6	HMF : Casein = 0: 100

Table 5

Group	Cholesterol Serum (mg/dl)
1	114±14 a
2	208±19 b
3	214±20 b
4	267±30 bc
5	381±57 cd
6	397±18 d

Mean±SE, n=6. Significant difference between different signs (p 0.05)

From the above data it was clear that: (1) HMF (No. 1) showed very excellent cholesterol-lowering effect, (2) this effect was proportional to the amount of HMF added, and (3) even if some HMF was replaced by casein it showed excellent cholesterol-lowering effect.

Example 3:

The separated soy protein (Fuji Pro NEW-R, Fuji Seiyu Kabushiki Kaisha)(100 weight parts) was dissolved in water to make a 10% solution (pH 2.0). One half part of pepsin (Difco Co.; 1: 10000, Bacto) was added and the protein was hydrolyzed at 37 °C for 17 hours. The hydrolysate solution was centrifuged (5,000 rpm x 20 min.) to get the insoluble fraction (ppt.). About two volumes of water added and washing done under stirring and the precipitate fraction obtained by another centrifugation was heated at 80 °C for 30 minutes to inactivate the enzyme. After lyophilization 30 parts of the cholesterol-lowering substance (HMF-2) was obtained. Fig. 3 shows the HPLC chromatographic pattern done as in Example 1. The molecular weight distribution was in the range of about 1,000-150,000 with the major peaks at MW of about 86,000, 250,000, 7,600 and 2,900. Table 6 shows the amino acid composition done as in Example 1.

Table 6. (Units in weight %)

Amino acid			Amino acid		
Thr		5.0	Trp	*	2.3
Tyr	*	4.0	H8s		2.6
Phe	*	6.3	Asp		9.1
Cys		1.8	Ser		5.4
Met		1.4	Glu		12.2
Val	*	6.4	Pro		4.4
Ile	*	5.9	Gly		5.2
Leu	*	11.0	Ala		5.9
Lys		5.2	Arg		5.8

From the said data it was learned that while in SPI the hydrophobic amino acid composition was 27.6 weight %, in HMF-2 it increased to 35.9 weight % (hydrophbic amino acids are indicated by *).

Example 4:

The cholesterol-lowering substance (HMF-2), prepared as in Example 3, was added to the high cholesterol diet (rf. Table 2) so it would be 20% of the nitrogen source, as prepared in Example 1. This diet was administered to Sprague-Dawley male rats (six animals/group) for 14 days, and as in Example 1, the cholesterol levels in liver and serum were determined by the enzymatic method. As control, Fuji Pro-R (Fuji Seiyu Kabushiki Kaisha), a separated soy protein, was used. Results are shown in Table 7.

Table 7

Group	Cholesterol	
	Serum (mg/dl)	Liver (mg/dl)
Control	291±11	45.5±2.2
HMF-2	109±5	6.98±0.7

From these data it is clear that the cholesterol-reducing substance (HMF-2) showed marked effect of reducing cholesterol level.

Brief Explanation of the Figures

Fig. 1 is the HPLC chromatogram of the cholesterol-reducing substance (HMF-2) at OD 280 nm obtained in Example 1.

Fig. 2 is the HPLC chromatogram of the cholesterol-reducing substance (purified HMF) at OC 280 nm obtained in Example 1.

Fig. 3 is the HPLC chromatogram of the cholesterol-reducing substance (purified HMF-2) at OD 280 nm obtained in Example 3.

Claims

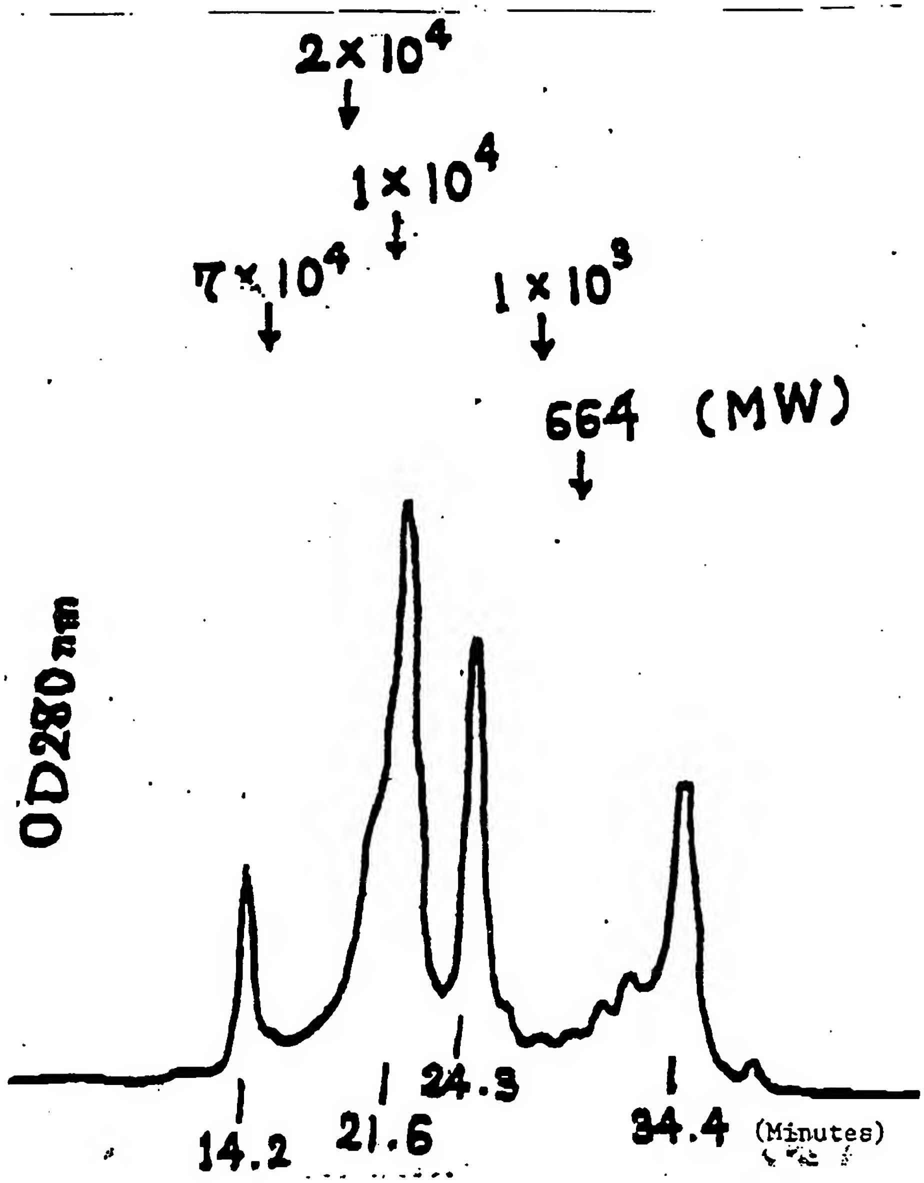
(1) Cholesterol-reducing substance which is a water-insoluble fraction of the protein hydrolysate freed from the water-soluble fraction(s) which is soluble in SDS (sodium dodecyl sulfate) solution and its molecular weight after dissolution in the SDS solution ranges from 1,000-150,000.

(2) In Claim (1) wherein the said protein is soy protein.

(3) In Claim (1) or (2) wherein the molecular weight of the water-insoluble fraction, after dissolution in 1% SDS solution, is in the range of 2,000-100,000.

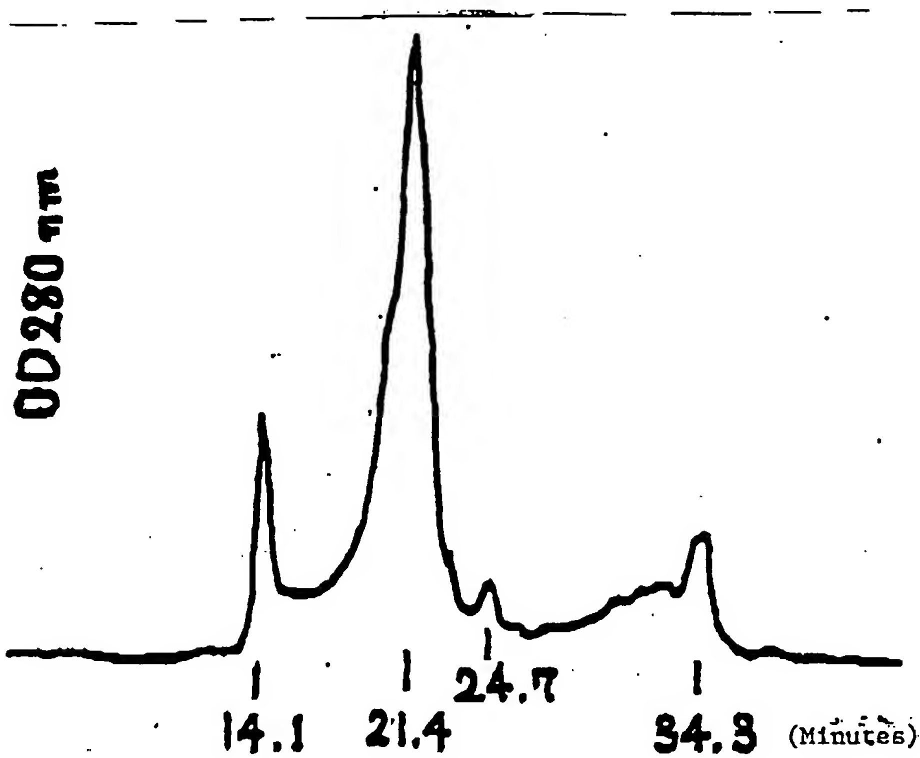
(4) In Claim (1) or (2) wherein the cholesterol-reducing substance has the hydrophobic amino acid composition in the overall amino acid composition over 30 weight % (preferably over 35 weight %).

Fig. 1



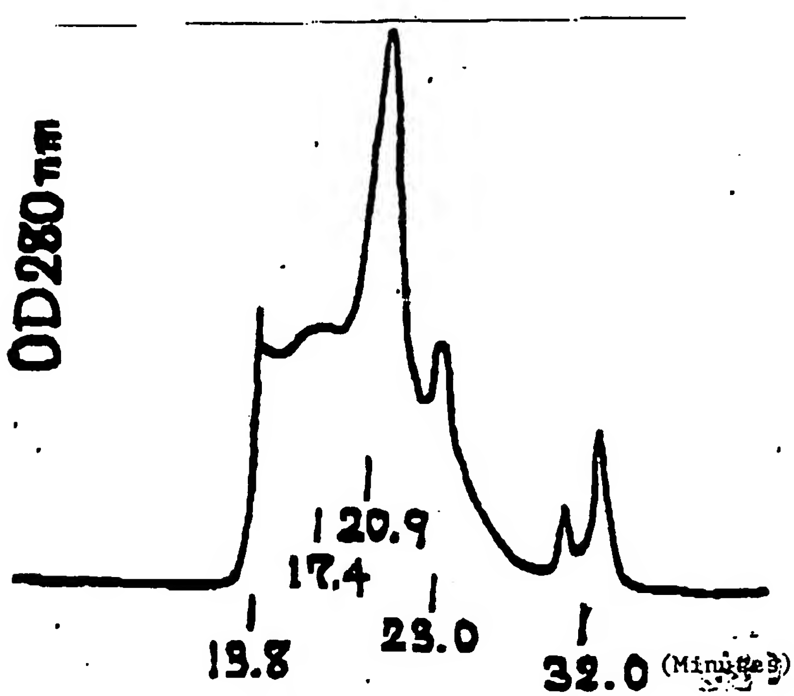
Elution time

Fig. 2



Elution time

Fig. 3



Elution time

INTERNATIONAL SEARCH REPORT

International Application No PCT/JP88/00781

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl[4] C07K15/02, 15/10, C12P21/06, A61K37/18, A23L1/305

II. FIELDS SEARCHED

Minimum Documentation Searched

Classification System	Classification Symbols
IPC	C07K15/02, 15/10, C12P21/06, A61K37/18, A23L1/305

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched

III. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	JP, A, 57-18995 (Terumo Corporation) 30 January 1982 (30. 01. 82) & US, A, 4452888	1-4
A	JP, A, 59-175437 (Teikoku Hormone Mfg. Co., Ltd.) 4 October 1984 (04. 10. 84) (Family: none)	1-4

* Special categories of cited documents:
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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
October 27, 1988 (27. 10. 88)	November 7, 1988 (07. 11. 88)
International Searching Authority	**Signature of Authorized Officer**
Japanese Patent Office	

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